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Michel Francis Bureau, Luc Wasungu, Lauriane Jugé, Daniel Scherman, Marie-Pierre Rols, et al.. Investigating relationship between transfection and permeabilization by the electric field and/or the Pluronic® L64 in vitro and in vivo.. The Journal of Gene Medicine, 2012, 14 (3), pp.204-15. 10.1002/jgm.2610 . inserm-00679701

HAL Id: inserm-00679701

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Submitted on 16 Mar 2012

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Investigating relationship between transfection and permeabilization by the electric field and /or the pluronic[®] L64, *in vitro* and *in vivo*

Short title : **Transfection vs permeabilization relationship**

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Abstract

Background

Electrotransfer can be obtained by the successive delivery of a high voltage short duration pulse (HV) inducing membrane destabilization then a low voltage long duration pulse (LV) allowing DNA electrophoresis (HVLV mode). Pluronic L64 (L64) has permeabilizing properties and amplifies expression of DNA. We wondered whether L64 could have an adjuvant effect on transfection by electrotransfer and whether the sequence L64 injection then application of a LV pulse could induce transfection comparable to that observed with the HVLV mode.

Methods

In vitro, we used fluorescence-activated cell sorting to evaluate the CHO cell transfection by a plasmid coding GFP, and permeabilization to propidium iodide. *In vivo*, the transfection efficiency of mice tibial cranial muscle was evaluated by optical imaging using a plasmid DNA encoding luciferase. For the same animals, permeabilization indices were evaluated by magnetic resonance imaging from the uptake of a T_1 contrast agent.

Results

Using the HVLV mode, transfection efficiency was low *in vitro* on CHO cells but high for muscles *in vivo*. Pre-treatment by L64 increased transfection efficiency of electrotransfer for CHO cells but not for the muscle. In mice muscles the L64 amplified the expression of DNA. Nevertheless, neither transgene expression nor permeability indices were further amplified by subsequent delivery of one LV pulse.

Conclusion

A major finding is that the nature of the membrane modification induced by electric pulses is not comparable to the one mediated by L64. The electrophoretic LV pulse does not induce additive effects to that of L64 for transfection improvement.

Key words: poloxamer; electrotransfer; MRI; luminescence; permeability; transfection

1 Introduction

Transfection of muscle using electric field application was developed in 1998 [1, 2] and is now a well-known, efficient procedure [3-6] but whose mechanisms remains largely unknown [7]. In addition, electrotransfer safety needs improvement, studies having shown that it induces an inflammation for 2 weeks after treatment [8] and can induce pain when high current intensities are applied [9, 10]. It has been proposed that electric field efficiency for DNA transfer through cell membranes results from two effects: (i) membrane modification as evidenced by its permeabilization to small molecules and (ii) DNA electrophoresis [11]. On the muscle model *in vivo* we have shown that the two effects can be combined by using the successive delivery of one high-voltage (HV) short-duration pulse, which is exclusively permeabilizing, and a low-voltage (LV) pulse of long duration, which is not permeabilizing but can induce DNA electrophoresis (HVLV mode) [12, 13]. Such a procedure is now also practically used for the transfection of other tissues than muscle [14-16]. More recently various copolymers of polyethylene oxide and polypropylene oxide have been shown to be efficient agents for improving muscle transfection [17-19] to a level comparable to that of electrotransfer [20]. These copolymers are chemically neutral and do not interact with DNA [21]. Physical studies with membrane models or liposomes showed that these copolymers interact with lipids with their more hydrophobic part, polypropylene oxide [21-23]. However, observation of labeled copolymers and DNA incubated with cells did not allow evidencing the copolymer at the level of the cell membrane, but rather inside the cells as macromolecular clusters [24, 25]. Moreover, copolymers were shown to improve transfection by acting on DNA nuclear import and its transcription through NFkB activation [26]. Among these copolymers, we chose the pluronic L64, which exhibits cell permeabilizing properties on artificial membranes [21, 27], on cells *in vitro* [28], and *in vivo* [20]. In a previous study, we had compared muscle transfection and permeabilization, either mediated by the pluronic L64 or electrotransfer [20]. In this study, we combined both pluronic L64 and electrotransfer. To obtain further insights, the present study aimed to determine whether pluronic L64, by its effects on nuclear import of DNA and its transcription, could have an adjuvant effect on transfection by electrotransfer. This had been shown on CHO cells *in vitro* [24], but had not been studied *in vivo* on a muscle model. This effect of pluronic L64 has also been demonstrated for *in vitro* transfection by a cationic polymer, polyethyleneimine [29].

Another question was does permeabilizing properties of the pluronic L64 can be compared to that of the electric field ?

It had been shown in previous studies on the muscle model that the order of the sequence HVLV was critical to amplify DNA transfection. Under conditions of these studies one HV or one LV pulse alone have no effect on DNA transfection as compared to simple DNA injection. For an efficient transfection amplification, DNA may be injected either before or after the HV pulse, and before the LV pulse, which is consistent with a direct effect of the LV pulse on DNA, i.e. electrophoresis [13]. It was also shown that the LV pulse was efficient only after the HV pulse had been applied, indicating that DNA electrophoresis is useful only when the cell membrane is in a modified state [12, 13]. Therefore, we investigated whether the same behaviour could be observed when the membrane modified state was induced by the pluronic L64. For that, we compared the HVLV mode to the delivery of pluronic L64 followed by a LV pulse (L64LV mode).

In summary we investigated, whether the pluronic L64 combined with electric pulses could improve gene transfection efficiency, and whether its permeabilizing property could be compared to permeabilization induced by electric field application. For this purpose we performed experiments *in vitro* on CHO cells and *in vivo* on muscle, in which transfection and permeabilization to small molecules were evaluated. In both cases, for the HVLV procedure HV was a brief high-voltage pulse, permeabilizing but not transfecting, and LV a long-duration pulse, not permeabilizing but allowing DNA electrophoresis.

2 Materials and Methods

2.1 Plasmid DNA

The pVAX2 construct was a pVAX1 plasmid (Invitrogen, Carlsbad, CA) in which the promoter was replaced by the pCMV β plasmid promoter (Clontech, Palo Alto, CA). The pVAX2-luc plasmid was a 4.6 kb vector encoding a cytosolic firefly luciferase plus protein (luc+) subcloned in the pVAX2 backbone. The expression vector pEGFP-C1 was a 4.7 kb plasmid containing the gene coding the Green Fluorescent Protein (GFP) under the control of the CMV promoter. It was obtained from Clontech. The plasmid DNA was purified from *E. coli* transformed cells using endo-free Qiagen Gigaprep kits (Qiagen, Courtaboeuf, France), and then diluted in saline (0.9% NaCl). The quality of the plasmid was assessed by calculating the ratio of light absorption (260 nm/280 nm) and by visualization on ethidium bromide-stained 1% agarose gel. Plasmid DNA was supercoiled for at least 80% of the preparation. Light absorption at 260 nm was used to determine DNA concentration.

2.2 Polymer

Pluronic L64 (Fluka, Sigma-Aldrich, L'Isle-d'Abeau Chesnes, Saint-Quentin Fallavier, France) is a copolymer of propylene oxide (PO) and ethylene oxide (EO). Its formula is $(EO)_{13}(PO)_{30}(EO)_{13}$. For *in vivo* experiments, pluronic L64 solutions were prepared on the day of the experiment as described previously [20]. The final injected solution (30 μ L) contained: 4.8 μ g of DNA, 3.33 μ moles of Gd-DOTA and 0 or 0.25% pluronic L64, according to the experiment. We chose the concentration of 0.25% in pluronic L64 because we previously showed that this concentration was optimal for transfection amplification in the mice muscle [20].

2.3 In vitro experiments

2.3.1 Cell culture

Chinese hamster ovary (CHO) cells were grown in Eagle's minimum essential medium (EMEM; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 8% fetal calf serum (Gibco), 100 units/ml of penicillin and 1 mg/ml of streptomycin (Gibco-Invitrogen), 0.58 mg/ml of L-glutamine (Eurobio, France) and vitamins (vitamin solutions from Gincor-Invitrogen, see manufacturer for composition). The cells were maintained in a 5% CO₂ humidified incubator at 37 °C (Jouan, Saint-Herblain, France).

2.3.2 Electric pulse delivery *in vitro*

Electrotransfection was performed using a CNRS cell electropulsator (Jouan, Saint-Herblain, France). Alternatively, for high-voltage and low-voltage electrical conditions a Cliniporator generator (IGEA, Carpi, Italy) was used. Stainless steel flat parallel electrodes 2 cm in length set 4 mm apart were used. For all experiments *in vitro* (as *in vivo*) electric field intensity was estimated as the voltage divided by the distance between the plate electrodes. Electropulsation was applied on 500,000 CHO cells suspended in 100 µl of pulsation buffer (10 mM K₂HPO₄/KH₂PO₄ buffer, 1 mM MgCl₂, 250 mM sucrose, pH 7.4, conductivity 1.6 mS/cm) to which was added 1 µl of plasmid DNA pEGFP-C1 solution (1 µg/µl). Several electrical parameters were tested as described in table 1. After pulsation the cells were transferred to a 35 mm Petri dish with 1.5 ml of growth medium and kept at 37 °C in a 5% CO₂ atmosphere.

For experiments with Pluronic L64 0.25%, cell pre-treatment, electropulsation was carried out on adherent CHO cells plated on a 24-well plate at a density of 50,000 cells per well 16 h before experiments, according to a procedure described previously [24]. Briefly, electropulsation was carried out directly in the well on adherent cells with stainless steel flat parallel electrodes (length 1 cm, set 1 cm apart). The electric field intensity used for optimal transfection was lower than for cell suspension because of the larger size of adherent cells. Conditions tested are described in table 1. For all experiments transfection efficiency was given as the percentage of fluorescent cells expressing the GFP and the average fluorescence level of positive cells was determined with a BD FACSCAN flow cytometer (BD Biosciences) 24 h after transfection.

2.3.3 Electroporabilization of cell suspension *in vitro*

Electroporabilization was studied with pulse conditions identical to the one used for electrotransfection of cells in suspension. The pulsation buffer contained 100 μ M of propidium iodide (PI, Sigma Chemical Co. St. Louis, USA) and no plasmid DNA. Directly after electropulsation, cells were transferred to 300 μ L of PBS and analyzed by flow cytometry with a BD FACSCAN flow cytometer (BD Biosciences). The efficiency of permeabilization was reported as the percentage of PI positive fluorescent cells and the average fluorescence level of these cells.

	Cell suspension 500 000 cells/100 μ L, 1 μ g DNA	Adherent cells 50 000 cells/well pretreated L64, 1 μ g DNA
Control : incubation with DNA only	Control	
ETs : 10 x 700 V/cm, 5 msec., 1 Hz	ETs	
ETa : 10 x 500 V/cm, 5 msec., 1 Hz		ETa
HV₁ : 1300 V/cm, 0.1 msec.	HV₁	
10HV₁ : 10 x 1300 V/cm, 0.1 msec., 1 Hz	10HV₁	
LV₀ : 80 V/cm, 200 msec.		LV₀
LV : 80 V/cm, 500 msec.	LV	LV
HV₁LV : HV then LV Delay between pulses 0.1 sec	HV₁LV	
10 HV₁LV : 10 HV then LV	10HV₁LV	

Table 1: Different modalities of electric pulses delivery used *in vitro*. For all experiments, temperature was set at 37°C.

2.3.4 Cell viability

Cell viability was measured by monitoring cell growth through coloration with crystal violet [30]. After staining with crystal violet and cells lysis the absorbance at 590 nm was measured with a spectrophotometer (Pharmacia Biotech). The viability was expressed by the percentage of coloration obtained as compared to cells treated in the same way but without application of an electric field.

2.4 In vivo experiments

2.4.1 Animals

In vivo studies were carried out on 6–8-week-old female BalbC/J mice (Janvier, Le Genest-Saint-Isle, France). Before all procedures (treatment and imaging), animals were anesthetized by intra-peritoneal injection of ketamine and xylazine (Bayer Pharma, Puteaux, France) (100 mg/kg and 10 mg/kg, respectively). The studies were conducted following the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimentation, and the local Ethics Committee on Animal Care and Experimentation.

2.4.2 General experimental procedure and electric pulse delivery *in vivo*

For each experiment, the tibial cranial muscle was injected longitudinally with 30 μ l of a solution containing DNA coding the firefly luciferase (4.8 μ g) and the Gd-DOTA (3.33 μ moles) with or without pluronic L64 (as previously described) by means of an insulin syringe (MYINJECTOR 29Gx1/2, Terumo, Leuven, Belgium). MRI measurements were performed at day 3 and optical imaging at day 7 after injection. In a few experiments only DNA was injected and consequently only measurement of transfection by optical imaging was performed. When required electric pulses were delivered 20 s after plasmid DNA injection through two stainless steel plate electrodes (10 \times 20 mm), placed 4 mm apart at each side of the mouse leg. Electrical contact with the shaved leg skin was ensured by means of a conductive gel (Aquasonic 100, Parker Laboratories, Fairfield, New Jersey, USA). Electric pulses were generated by a Cliniporator electropulsator (IGEA, Carpi, Italy). Several electrical parameters were tested as described in the table 2. Our procedure of electrotransfer ET that we named standard was used as an efficient condition in many of our previous studies [12, 13, 20]. The HVLV condition was derived from our study of 2002 [13] but with a LV of 500 msec. duration in order to accentuate electrophoretic effect. These optimal conditions for the muscle are different from those used on CHO cells *in vitro* because of a different threshold for permeabilization. Indeed we have shown in a previous study that 8 pulses of 600 V/cm (1 msec.) at 1 Hz were lesional for the muscle. Thus 10 pulses of 700 V/cm 5 msec. at 1 Hz are not adapted to muscle transfection whether they are optimal for CHO cells [31].

Tibial cranial muscle 4.8 μg DNA + 3.33 μmoles Gd-DOTA MRI and Optical imaging	Tibial cranial muscle 4.8 μg DNA Only optical imaging
ET : 8 x 190 V/cm, 20 msec., 2 Hz	ET120 : 8 x 120 V/cm, 20 msec., 2 Hz
HV : 800 V/cm, 0.1 msec.	HV : 800 V/cm, 0.1 msec.
LV : 80 V/cm, 500 msec.	L64ET120 : injection of pluronic L64 with DNA 15 minutes before ET120
HVLV : HV then 0.5 sec later LV	L64HV : injection of pluronic L64 with DNA just before HV
L64 : injection of pluronic L64 with DNA	L64 : injection of pluronic L64 with DNA
L64LV : injection of pluronic L64 with DNA just before LV delivery	DNA : simple DNA injection
DNA : Simple DNA injection	

Table 2: Different modalities of electric pulses delivery used in vivo.

2.4.4 MRI acquisition

MRI examinations were performed with a 7 T vertical wide-bore magnet (Bruker) equipped with a standard proton micro-imaging probe and ParaVision 2.1.1 software. Mice were housed and kept upright using a custom-made support of diameter 38 mm. The two hind limbs were positioned symmetrically and examined simultaneously in transversal planes using a T_1 -weighted spin-echo sequence with the following parameters: repetition time (T_R) 600 msec., echo time (T_E) 10 msec., 12 contiguous slices of thickness 1 mm, field of view 30 mm, spatial resolution $117 \times 117 \mu\text{m}^2$ in-plane and acquisition time 20.3 min. The slice centered on the largest signal of interest was chosen for T_1 and T_2 measurements, using a single slice spin-echo sequence with the following parameters: T_R 3000, 2000, 1200, 800, 600, 500, 400 and 300 msec., eight echoes, first echo at T_E 9 msec., echo spacing 8 msec., field of view 30 mm, in-plane spatial resolution $117 \times 234 \mu\text{m}^2$, section thickness 1.5 mm and acquisition time 18.5 min.

2.4.5 MRI processing

The assay Gd-DOTA trapped in tibialis muscle was obtained from T_1 -weighted images.

We used two parameters to characterize this uptake: the volume of enhanced T1 MRI signal (permeabilized volume) and the concentration of the contrast agent Gd-DOTA in this volume, calculated from T_1 values. In brief, the permeabilized volume is correlated to the number of muscle fibers that are permeabilized, and the concentration of contrast agent to the amount of compound taken up by those fibers.

The volume labeled by the Gd-DOTA and the concentration of Gd-DOTA in this volume were evaluated as described previously [20].

2.4.6 *In vivo* optical imaging of luciferase activity

Luciferin potassium salt (SYNCHEM, Felsberg/Altenburg, Germany) diluted in PBS was injected locally into the tibial cranial muscle at a dose of 100 $\mu\text{g}/40\text{ }\mu\text{l}$, which is in large excess relative to the amount of luciferase [32]. Optical imaging was performed with a cooled intensified charge-coupled device (CCD) camera (Biospace, Photo Imager, Paris, France) placed in a black box. Luminescence level was measured in ROI corresponding to the tibial cranial muscle as described previously [20]. ROIs were very similar from one experiment to another. We opted to take the mean values in cpm of all the measurements for 10 min after the start of acquisition [32].

2.4.7 Statistical analysis of *in vivo* results

Because of the non-Gaussian distribution of the results, we used nonparametric tests: Kruskal-Wallis nonparametric variance analysis of the measured parameters and pairwise Wilcoxon test for comparisons between treatments. In addition, we used the Spearman correlation test to check for correlations between some parameters. Statistical program used were Statview (SAS Institute inc) and R (the R foundation for Statistical Computing).

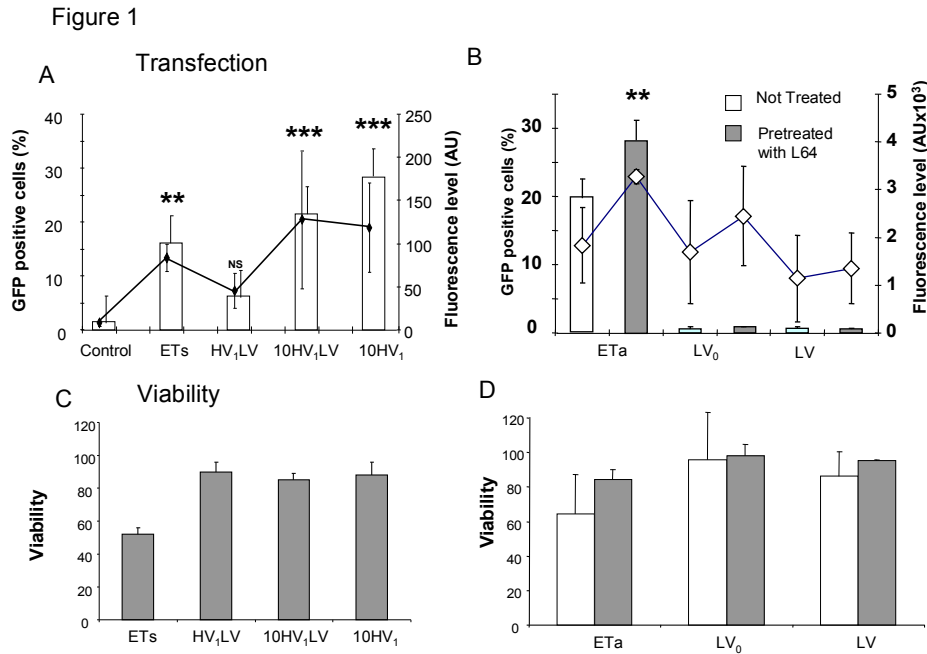
3 Results

First transfection and permeabilization experiments were performed *in vitro* on CHO cells then *in vivo* on the mice tibial cranial muscle.

3.1 *In vitro* CHO model

3.1.1 Transfection and cell viability

As expected, electrotransfer applied in appropriate conditions for CHO cells in suspension (ETs: 10x700 V/cm, 5 msec.) allowed for a significant transfection. However, the pulse combination HVLV mode, which was shown to be very efficient for muscle transfection, had a low efficacy on CHO cells *in vitro* (Fig. 1A) even though HV₁ (1300 V/cm, 0.1 msec.) electric field intensity was well above the threshold to permeabilize cells [33] and LV (80 V/cm, 500 msec.) was at a level and duration sufficient to induce DNA electrophoresis [34].



However using 10 HV₁ pulses allowed a significant transfection which was not obtained with one HV₁ (data not shown). The addition of a LV pulse to the 10 HV₁ pulses did not improve the transfection. The patterns for the percent of cell transfected or the mean fluorescence intensity per cell were similar. Co-incubation of DNA and pluronic L64 with CHO cells did not result in cell

transfection (data not shown), as observed by other authors with COS7 cells [18]. However, pre-treatment of adherent CHO cells with pluronic L64 at 0.25 % improved transfection by electrotransfer (Eta: 10x500 V/cm, 5 msec., 1 Hz) (Fig. 1 B). But, this pre-treatment followed by delivery of a low voltage pulse of long duration (80 V/cm, 200 or 500 msec.) did not induce a detectable transfection. Also, as expected, LV alone did not induce transfection.

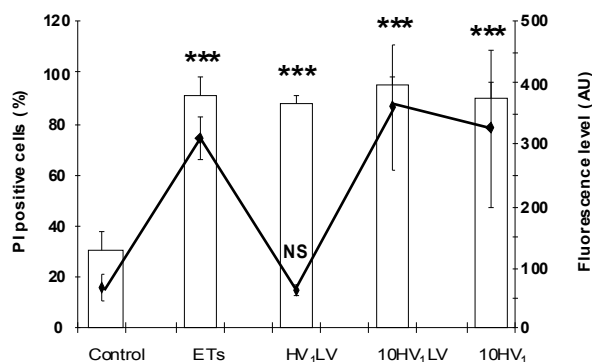
For all these experiments, cell viability was of about 60 % after electrotransfer (ETa or ETs) and more than 80 % for all other conditions (Fig. 1C and 1D).

3.1.3 Permeabilization

The percentage of cells permeabilized *in vitro* (Fig. 2) increased after ETs or pulse combination HV₁LV. As expected, LV did not increase the percentage of cells permeabilized to propidium iodide (data not shown).

Figure 2

Permeability



The mean propidium iodide intensity in cells was much lower after HV₁LV than after ETs for both series of experiments (Fig. 2).

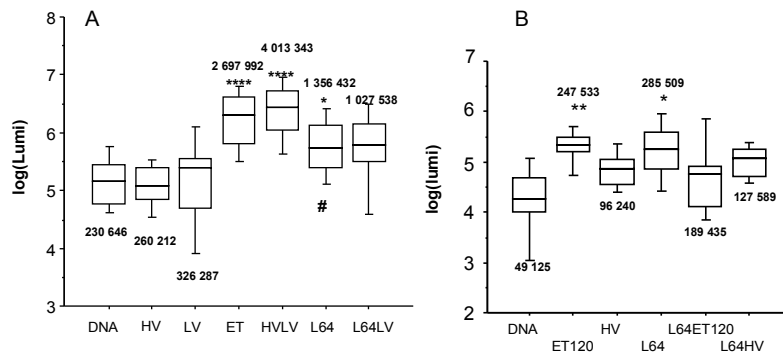
We did not study the permeabilizing effect of pluronic L64 *in vitro*, since it had no effect on transfection when co-incubated with DNA and CHO cells (data not shown). However, such a permeabilising effect *in vitro* had been shown previously [35]. Also, we have not studied the permeabilization to propidium iodide under the conditions of figure 1B, since we had previously shown that pre-treatment with 0.05 % pluronic L64 was not related to an increased permeabilization [24].

3.2 *In vivo* muscle model

3.2.1 *In vivo* muscle transfection

Previous studies had shown that *in vivo* luminescence was a reliable index of the luciferase reporter gene transfection level [32, 36]. As shown in Fig. 3A, with an injected amount of 4.8 μ g DNA, the luciferase transgene expression (luminescence) was significantly increased by either standard electrotransfer (ET, 8×190 V/cm, 20 msec., 2 Hz) or pulse combination (HVLV: HV 800 V/cm, 0.1 msec. then LV 80 V/cm, 500 msec.) at a comparable level, relative to the simple DNA injection (DNA). As expected, electrotransfer with HV or LV alone had no significant effect on transfection level (Fig. 3A HV, LV). Pulse combination with a LV of 300 msec. was less efficient than with an LV of 500 msec. (data not shown). Luminescence after DNA co-injection with pluronic L64 at 0.25% was also significantly increased relative to luminescence after simple DNA injection (Fig. 3A, L64). However, in this group of experiments this luminescence value was significantly lower than after pulse combination (HVLV). Delivery of one LV pulse after co-injection of DNA and pluronic L64 had no effect on the transfection level (Fig. 3A, L64LV). We hypothesised that pluronic L64 muscle fiber internalisation and consequently its amplification effect on DNA expression could be favored by a permeabilizing HV pulse. However, delivering an HV pulse before or after co-injection of DNA and pluronic L64 had no detectable transfecting effect. Similarly, combining LV or HV pulse with a lower concentration of 0.1% pluronic L64 had no transfecting effect (data not shown).

Fig 3



In order to know if as *in vitro*, *in vivo* pre-treatment of muscle with pluronic L64 can improve transfection level we performed another series of experiments (Fig 3B). We assumed that with a mild electrotransfer condition (8x120 V/cm, 20 msec., 2 Hz) amplification effect of the pluronic L64 on DNA expression could be more easily detected. However, pluronic L64 was unable to improve transfection when injected 15 minutes before DNA and a mild electrotransfer condition ((Fig. 3 B, L64ET120) or an HV pulse (Fig. 3 B, L64HV).

3.2.2 *in vivo* muscle permeabilization

3.2.2.1 - Distribution of the Gd-DOTA in the tibial cranial muscle

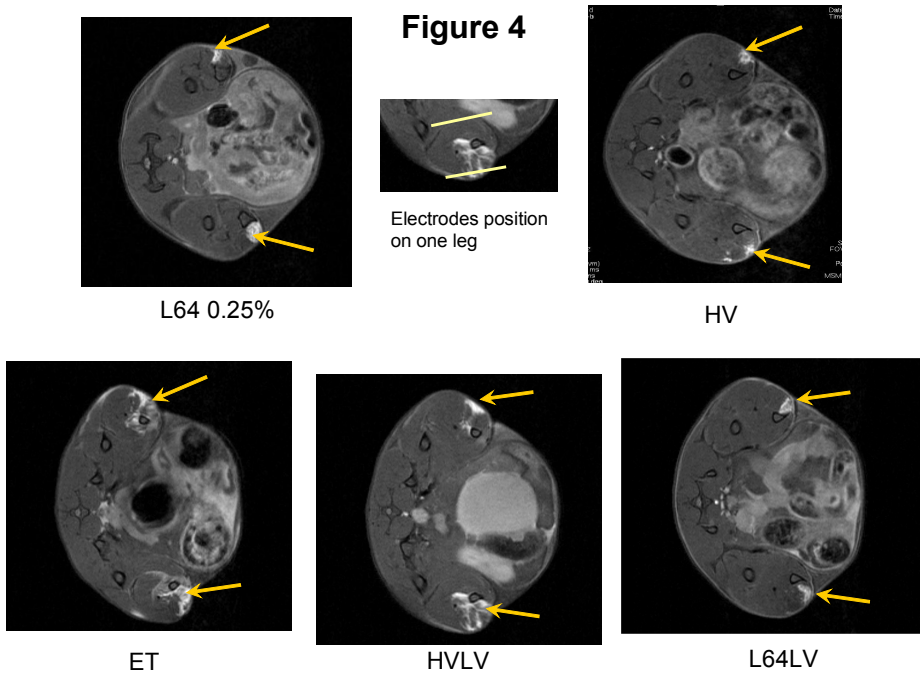


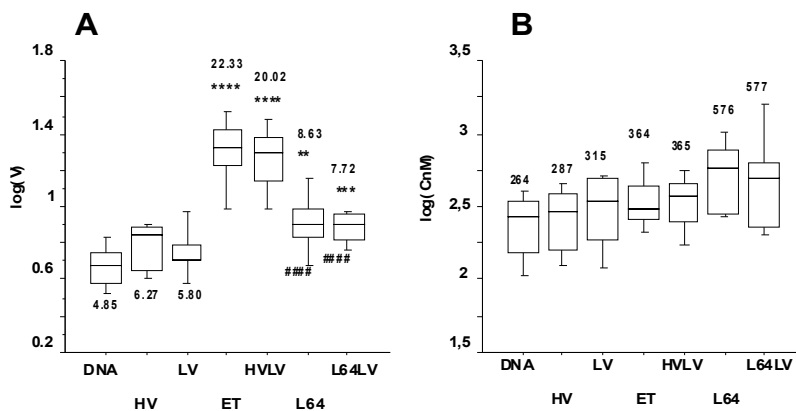
Figure 4 shows characteristic T_1 -weighted images. Gd-DOTA as DNA and pluronic L64 were injected locally into muscle (see Materials and Methods). We see that the distribution of the contrast agent in the tibial cranial muscle zone is localized and relatively homogenous after pluronic L64 (0.25 %) or HV treatment. By contrast, this distribution of the Gd-DOTA uptake is heterogeneous and enlarged after standard ET as well as after HVLV delivery. For L64 (0.25 %) injection then LV application distribution of Gd-DOTA seems similar to the one obtained with L64 alone.

3.2.2.2 - Permeabilized volume (V_{Gd})

As shown in Figure 5A, relative to the simple DNA injection, pulse combination (HV 800 V/cm, 0.1 msec. then LV 80 V/cm, 500 msec.: HVLV), standard electrotransfer (8 × 190 V/cm, 20 m, 2 Hz: ET) and DNA co-injection with pluronic L64 at 0.25% (L64) induced a significant increase in the volume permeabilized to Gd-DOTA. This increase in the permeabilized volume was higher for standard electrotransfer or HVLV relative to that induced by DNA co-injection with pluronic L64. Electrotransfer with HV alone induced a slight, non significant increase in the permeabilized volume. Just as for transgene expression (luminescence), the use of LV alone had no significant effect, nor did the delivery of one LV pulse after co-injection of DNA with pluronic L64 0.25%. Similarly, we did not observe any effect on the permeabilized volume of HV or LV combination with pluronic L64 0.1% (data not shown).

3.2.2.3 - Concentration of the Gd-DOTA in the permeabilized volume (C_{Gd})

Effects of the different treatments on the concentration of the contrast agent (C_{Gd}) in the permeabilized volume were less obvious. Mean values for ET, HVLV and pluronic L64 were higher than for simple DNA injection, but not significantly. Just as for the permeabilized volume and transfection level, the C_{Gd} after DNA coinjection with pluronic L64 0.25 % was not modified by the delivery of an LV pulse (Fig. 5 B).

Figure 5

3.2.3 Relations between “permeabilization” and transfection

With the muscle model *in vivo* it was possible to study statistically the relations between transfection and permeabilization parameters measured on the same mice [20].

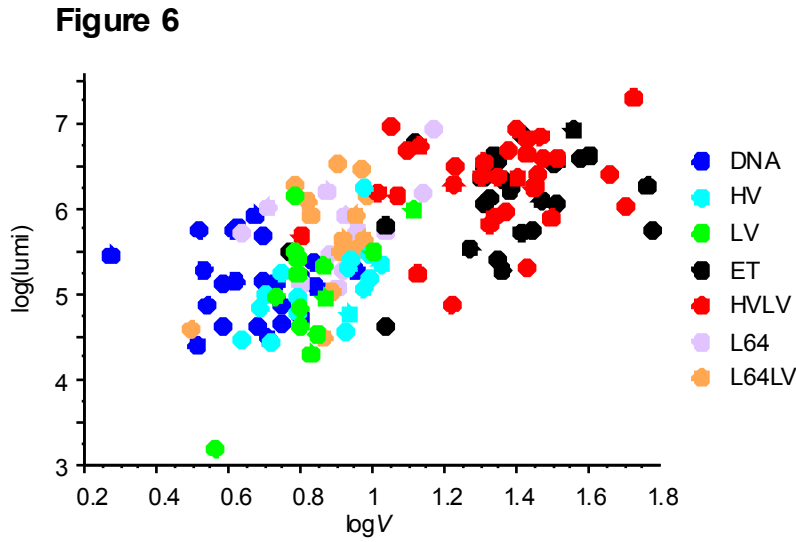
The strength of the correlation between the permeabilized volume to Gd-DOTA (V_{Gd}) or the concentration of Gd-DOTA in this volume (C_{Gd}) and transgene expression (luminescence) was established using the nonparametric Spearman test. From this test, considering all studied groups, we found a correlation coefficient (ρ) between luminescence and V_{Gd} of 0.718, which was statistically very significant ($p < 0.0001$), as shown in Table 3. To improve the analysis, we treated different sub-groups separately. These results show that with three modalities of DNA transfection inducing muscle fibre permeabilization, i.e L64, ET, HVLV, the level of transfection was correlated to the volume permeabilized to Gd-DOTA. The correlation with the concentration of Gd-DOTA in the permeabilized volume was lower but also significant.

Table 3. Correlations between luminescence and volume permeabilized to Gd-DOTA (V_{Gd}) or concentration of Gd-DOTA in the permeabilized volume (C_{Gd}). V2

Treatment group	Correlation coefficient (ρ)	
	V_{Gd} vs. luminescence	C_{Gd} vs. luminescence
All groups $n = 139$	0.713 ($p < 0.0001$)	0.336 ($p = 0.0022$)
DNA, L64, L64LV. $n = 49$	0.443 ($p = 0.0022$)	0.449 ($p = 0.0019$)
DNA, ET, HVLV $n = 81$	0.644 ($p < 0.0001$)	0.368 ($p = 0.0009$)
DNA, ET. $n = 51$	0.713 ($p < 0.0001$)	0.436 ($p = 0.0021$)
DNA, HVLV $n = 55$	0.706 ($p < 0.0001$)	0.328 ($p = 0.0160$)
DNA, HV, LV $n = 53$	0.209 ($p = 0.1314$)	0.345 ($p = 0.0128$)

Rho: strength of the link between the paired values (0 no relation, 1 and -1 perfect correlation; 1: direct, -1 : inverse); n : number of paired values; p : statistical significance.

Fig 6 provides a graphic depiction of the relation between luminescence, our index of muscle transfection and the muscle volume permeabilized to Gd-DOTA (V_{Gd}). Top right: scatter plot for electrotransfer by the standard (ET) or pulse combination (HVLV) procedure. Bottom left: scatter plot for DNA injection or DNA injection followed by an HV or LV pulse. Center: scatter plot for DNA coinjection with pluronic L64 followed or not followed by an LV pulse.



4 Discussion

In this study we compared transfection and permeabilization *in vitro* and *in vivo* with different modalities of electric pulse delivery with or without association of pluronic L64 (L64). Our *in vitro* model was CHO cells and the *in vivo* model was the mouse tibial cranial muscle. In both models we confirmed a high level of transfection associated with permeabilization to small molecules with a series of electric pulses in conditions previously defined [11, 33], which we term standard electrotransfer (ET). However, using a combination of one high-voltage short-duration (HV) and one low-voltage long-duration (LV) pulses, the HVLV mode [12, 37], we found that transfection efficiency was low *in vitro* on CHO cells but high for muscles *in vivo*, at a level similar to standard electrotransfer ET.

We will first discuss the combination of HV and LV pulses and then go on to analyze results for the association of pluronic L64 and the electric field.

4.1 HVLV *in vitro*

In vitro, HV₁LV did not induce many transfected cells even though a great number of cells were permeabilized to propidium iodide (PI). However, the mean PI fluorescence level per cell was much lower than with the ETs condition. In these conditions DNA electrophoresis by LV is ineffective and thus pointless. Other studies showed an effect of HVLV mode *in vitro*. Sukharev et al [37] who proposed the pulse combination (HVLV) approach showed electrotransfection efficiency on a different cell type (simian cos cell) and with very different parameters (HV : 4-7 kV/cm, 10-20 μ sec; LV 0.2-0.5 kV/cm, 1-10 msec.). Cepurniene et al with conditions closer to ours, found a low percent of GFP positive cells, which is similar to our results [38]. Using a more sensitive reporter gene such as luciferase they revealed a clear effect of HVLV on transfection. Although the detection sensitivity of transfected cells could be improved with HVLV in the present study, the mean level of “permeabilization” per cell remained low compared to the standard condition ETs (10 x 700 V/cm, 5 msec., 1 Hz) (see Fig 1). In summary, the high number of weakly permeabilized cells after one HV₁ pulse (1300 V/cm, 0.1 msec.) was not increased by adding one LV pulse (80 V/cm, 500 msec.) and nor was the permeabilization level. In a study of Pavlin *et al.* the high voltage treatment is given by four HV pulses of 200 μ s duration with amplitude of 1.0 kV/cm [39]. In our study increasing the number of HV₁ pulses to 10 allowed inducing a clear cell transfection associated to increased permeabilization. Application of one LV pulse after the 10 HV₁ pulses had no significant effect either for permeability or for transfection. This lack of effect could be related to the level of DNA concentration. Indeed, it was shown with CHO cells that transfection obtained with HV pulses could be increased by a subsequent LV pulse only at DNA concentration below 10 μ g/ml [39, 40]. For higher DNA concentration, electrophoretic improvement might not be needed as a result of there being sufficient DNA interaction with the cell membrane.

4.2 HVLV *in vivo*

We had shown in previous studies on muscle, and confirm here, that one HV (800 V/cm, 0.1 msec.) or one LV pulse (80 V/cm, 500 msec.) alone does not greatly improve transfection relative to simple DNA injection [12, 13]. The high transfecting condition HVLV led to a significant increase in the volume permeabilized to the contrast T1 agent Gd-DOTA (V_{Gd}). The concentration of Gd-DOTA (C_{Gd}) in this volume was increased but non significantly. Overall, the uptake of Gd-DOTA ($V_{Gd} \cdot C_{Gd}$) was increased. These results mean that LV not only induce DNA electrophoresis but also enhances the permeabilizing effect of HV as observed in our previous studies [12, 13].

It is not possible here to determine whether the increased uptake of Gd-DOTA is related to an increase in the membrane permeability coefficient or to a longer duration of the permeabilized state. However, in one study based on the pore theory of electroporation it was suggested that in the pulse combination procedure (HVLV) the long-duration low-voltage pulse LV could stabilize the pores created by the HV pulse [41].

4.3 HVLV *in vivo* vs. *in vitro*

A better transfection in muscle by HVLV compared to that obtained *in vitro* may result from the additive effect of LV on membrane permeabilization by HV. The required LV field intensity *in vitro* on CHO cells to obtain synergy between HV and LV for increasing membrane permeabilization may need to be higher. This seems possible since the threshold electric field intensity to permeabilize CHO cells is close to 300 V/cm [33] whether it would be close to 100 V/cm for muscle fibers [11]. To note that for muscle fibers a threshold of 200 V/cm was found with 8 pulses of 0.1 msec. at 1 Hz [42]. However, it was shown on CHO cells that the necessary electric field intensity for electroporation of a given fraction of cells was decreasing with pulses of longer duration [43]. Consistently, in the present study delivering 8 pulses of 190 V/cm and 20 msec. duration at 2 Hz was clearly above the threshold for permeabilization and transfection.

Another hypothesis is that injected DNA that is transfected is in a particular compartment [44] that may be close to fiber membrane (as in T tubule....) [45]. Also, cell nuclei are closer to the cell membrane than for CHO cells. Thus small electrophoretic movements of DNA induced by LV may be more efficient than *in vitro*. It was shown *in vitro* that LV pulse after HV pulse increased transfection efficiency only for low concentration [40]. It is assumed by the authors that there is low local concentration *in vivo*. However the plasmid solution injected into muscle was highly concentrated (160 µg/ml) and we do not know how DNA is distributed in this tissues.

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4.4 Pluronic L64 *in vivo* vs. *in vitro*

The purpose of our study was also to compare electric field effects with those of pluronic L64 and their combination. From the literature, it is known that pluronic L64 is inefficient *in vitro* but allows muscle transfection to be improved *in vivo* when co-injected with DNA [18], as confirmed by our study. Thus, it was shown to improve gene expression *in vivo* in the muscle where a basal transfection occurs by simple DNA injection [46]. The proposed mechanism is that pluronic L64 favors nuclear import of cytoplasmic DNA and its transcription via activation of the NFκB pathway [29]. For this to happen it is necessary that DNA crosses the plasma membrane. This

occurs in the muscle, since a simple DNA injection allows fiber transfection [46]. However, this apparently does not occur *in vitro* by coincubation of DNA and pluronic L64 with CHO cells. It is also necessary to have DNA with consensus sequences for NFkB, which was shown for the CMV promoter of plasmid DNA that we used [47].

4.5 Pre-treatment with Pluronic L64 vs. ET

One of our aims in undertaking this study was to assess the possibility that the pluronic L64, by its effects on nuclear import of DNA and its transcription, could amplify transfection by electrotransfer in muscle *in vivo* as we have shown *in vitro* with CHO cells in a previous study [24]. *In vitro* pre-treatment of CHO cells with pluronic L64 0.25 % allowed improvement of transfection by standard electrotransfer (10 x 500 V/cm, 5 msec., 1 Hz) whereas *in vivo*, muscle pre-treatment with 0.25% before a mild electrotransfer (8 pulses 120 V/cm, 20 msec., 2 Hz) did not improve transfection. A first hypothesis is that interaction of pluronic L64 with muscle fiber membrane and its subsequent internalization is not the same as with CHO cells. Another hypothesis is that electrotransfer alone is able to activate the NFkB pathway. Consequently, any additional effect of pluronic L64 is reduced or null. Such an activation of NFkB by ET is possible, since it is known that this procedure induces an inflammation [8, 20, 48], and thus an activation of the NFkB pathway [49]. In our previous *in vitro* study we observed that electrotransfer was more efficient using a plasmid with NFkB consensus sequences [24] (see Fig 6 of this article). Lastly, a slight improvement in transfection by pluronic L64 as observed with CHO cells *in vitro* (less than twofold increase) is difficult to detect *in vivo* owing to a variation of about one log between data in the same group.

4.6 Comparison between membrane destabilization with Pluronic L64 and HV

Another question was whether membrane destabilization induced by pluronic L64 could be equivalent to that induced by HV, and consequently whether we could evidence any synergy between pluronic L64 and LV as we observed between HV and LV for transfection and permeabilization of the muscle. We found that pre-treatment by pluronic L64 before an LV pulse was inefficient both *in vitro* and *in vivo*. *In vitro* it could be expected since LV or pluronic L64 alone does not lead to DNA transfection. *In vivo*, the delivery of one LV pulse after pluronic L64 administration did not further amplify the transfection level, the muscle volume permeabilized to Gd-DOTA and the concentration of Gd-DOTA relative to pluronic L64 alone, in contrast to what was observed with delivery of LV after HV. It is thus clear that pluronic L64 do not induce

membrane modifications similar to those obtained by the delivery of an HV electric pulse. Even though, pluronic L64 is able to create pores in phospholipid membranes, this destabilisation is more than likely not of the same nature as the one by an electric field. First pluronic L64 might cover the phospholipid heads, masking the charges at the surface of the membrane. Second, insertion of pluronic L64 might not induce charge distribution modification as opposed to electrotransfer.

We showed in our previous study [24] that on CHO cells *in vitro*, pluronic L64 induced permeabilization to propidium iodide (PI), but at a lower level than with ETa (10 pulses of 500 V/cm, 5msec. at 1 Hz). In pre-treatment experiments, i.e CHO cells incubated with pluronic L64 followed by ETa, the permeabilization to PI was similar to that obtained with ETa alone. This confirms that the nature of membrane modification induced by electric pulses is therefore not comparable to that obtained by pluronic L64, and these effects are not additives. Alimi-Guez *et al.* also showed that gene transfection was not related to membrane permeabilization using non-permeabilizing copolymer [28]. In addition, other authors have shown that permeabilization induced by cationic polymer or cationic lipids is a side effect not related to transfection [50]. DNA electrophoresis by LV could improve transfection by favoring DNA interaction with the membrane or its movement through the membrane. As shown *in vitro* it seems that DNA interaction with the membrane is favored by electrophoresis for a given state induced by the electric field [51]. Our experiment shows that such a state cannot be induced by the pluronic L64. Another effect of DNA electrophoresis on transfection could be to modify its biodistribution in such a way that it would reach a higher concentration near the membrane. We have seen that this effect was non significant *in vitro* with the DNA concentration used. *In vivo*, in the muscle such an effect was also non detectable. Indeed LV did not improve significantly DNA transfection.

4.7 Correlations between transfection and permeabilization indices

In vivo, we observed a positive correlation between the permeabilized volume V_{Gd} and transgene expression (luminescence) for pluronic L64 and for ET (8 pulses of 190 V/cm and 20 msec. at 2 Hz) confirming our previous results [20]. The same significant correlation was evidenced with HVLV. Permeabilization to small molecules is an index of membrane modification associated with transfection. But DNA transfer probably does not occur by passive diffusion as it does for small molecules. *In vitro* we had no such possibility of statistical analysis. However, we observed that a minimal level of membrane modification, as evidenced by its permeabilization to propidium iodide, was necessary for transfection by electric pulse delivery. From present

knowledge on *in vitro* gene transfer it is also probable that electroporation does not allow DNA transfer by passive diffusion [7].

In conclusion, the main finding of the present study is that the nature of membrane modification induced by permeabilizing electric pulses differs from that induced by pluronicL64. The electrophoretic LV pulse does not induce additive effects to the transfection mediated by pluronic L64. This indicates that DNA electrophoresis would only favour DNA interaction with membranes modified by permeabilizing electric pulses but not with membranes permeabilized by pluronic L64. In addition, the possible effect of DNA electrophoresis on its tissue biodistribution had no significant effect.

Acknowledgements: This work was supported in part by a grant from Agence Nationale pour la Recherche (ANR PCV06 139888), CNRS and INSERM French National Research. We are grateful to Julie Orio and Laetitia Helaudais for their assistance with some *in vitro* experiments.

The authors declare no conflict of interest.

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Figures Captions

Figure 1

CHO cells in vitro. Effect of different electric pulse delivery modalities and of pre-treatment with pluronic L64 on Transfection and Viability.

CHO cells in suspension or adherent were electrotransfected with a plasmid coding the Green Fluorescent Protein (GFP).

Group designations on the abscissa are for ETs: 10 pulses of 700 V/cm , 5 msec. duration at 1 Hz. ETa: 10 pulses of 500 V/cm, 5 msec. at 1 Hz. HV₁: 1 pulse of 1300 V/cm, 0.1 msec..

HV₁LV: the combination of high voltage and low voltage electrical conditions with LV: 1 pulse of 80V/cm, 500 msec.. LV: low voltage alone. 10 HV₁LV: ten HV₁ pulses then one LV pulse. 10 HV₁: ten HV₁ pulses. The control group corresponds to simple DNA incubation with CHO cells.

LV₀: 1 pulse of 80 V/cm, 200 msec..

Transfection

Panel A cell suspension

The percentage of GFP positive cells is represented with histograms on the left axis. This is the percent of viable cells transfected. The level of fluorescence (AU: arbitrary units) in the population of fluorescent cells is represented with squares on the right axis. Each experiment was performed three times in triplicate, and a minimum of 5000 cells were counted with cytometry.

Six to nine experiments were performed and four for control. The error bars are the standard deviation. Statistical comparisons between the different treatments were made using a PLSD Fisher test. Significance of the difference relative to control for the percent of GFP positive cells and the mean fluorescence intensity per cell: ** $p < 0.01$ *** $p < 0.001$

Panel B adherent cells

Before transfection adherent cells were treated (grey histograms) or not treated (white histograms: control) with 0.25% L64. The percentage of GFP positive cells is represented with histograms on the left axis. The level of fluorescence (AU: arbitrary units) in the population of fluorescent cells is represented with squares on the right axis. Error bars represent the standard deviation. Each experiment was performed from two to four times in duplicate and a minimum of 5000 cells were counted by cytometry. The error bars are the standard deviation. Statistical comparisons between the different treatments were made using a PLSD Fisher test. For electrotransfer we observed a significant difference between cells pretreated by the pluronic L64 0.25 % and cells not pretreated for the percent of GFP positive cells ** $p < 0.01$.

Cell viability

Column levels represent the mean percent of viable cells relative to the total number of cells. The error bars are the standard deviation. N=6 to 9.

Panel C cells suspension

Panel D adherent cells: Gray column design experiment without pluronic. White column design experiments with adherent cells pretreated by pluronic L64, 16 h before electric pulses delivery.

Figure 2

Permeabilization of CHO cells to propidium iodide

CHO cells in suspension were electroporated in the presence of the fluorescent probe propidium iodide (PI). Several electrical conditions were tested: standard electroporation (ETs: 10 pulses of 700 V/cm, 5 msec, 1 Hz), high voltage electrical parameters (HV₁: 1 pulse of 1300 V/cm, 0.1 msec.), the combination of high voltage and low voltage electrical conditions (HV₁LV, LV: 1 pulse of 80V/cm, 500 msec.) and finally low voltage alone (LV). Non treated cells which are only incubated with PI are represented as control. The percentage of PI positive cells is represented with histograms on the left axis. The level of fluorescence (AU: arbitrary units) in the population of fluorescent cells is represented with squares on the right axis. Values are the mean of 3 to 9 experiment performed in triplicate and a minimum of 5000 cells were counted by cytometry. The error bars are the standard deviation. Significance of the difference relative to control for PI positive cells and the mean fluorescence intensity per cell: *** $p < 0.001$. The level of fluorescence did not differ significantly from the control for the HV₁LV group.

Fig 3

Effect of pluronic L64 and / or electric field on transfection levels *in vivo*.

The distribution of values for each treatment are represented by box plot i.e out of the box smallest and maximum values (10 % and 90 %) then in the box the lower quartile, median and upper quartile.

A Tibial cranial muscle *in vivo*. Effect of different electric pulse delivery modalities on transfection . The mean value of the luminescence in cpm/min is given near each box. Group designations on the abscissa are for injection of free DNA (DNA); DNA then HV (HV); DNA then LV (LV); DNA then ET (ET); DNA then HVLV (HVLV); DNA and L64 0.25% (L64); DNA and L64 0.25% then LV (L64LV). With HV 800 V/cm, 0.1 msec., LV 80 V/cm, 500 msec.; HVLV one HV pulse followed 0.5 s later by one LV pulse; ET 8 pulses of 190 V/cm, 20 msec. at 2 Hz. The number of muscle for each treatment is of 12 to 33. Statistical significance of the difference relative to injection of free DNA : **** $p < 0.0001$, * $p < 0.05$; relative to HVLV: # $p < 0.05$

B Tibial cranial muscle *in vivo*: Effect of muscle pre-treatment with L64 0.25% injection before a mild electrotransfer or an HV pulse. The mean value of the luminescence in cpm/min is given near each box. Group designations on the abscissa are for injection of free DNA (DNA); DNA then mild electrotransfer (ET120); DNA then HV (HV); DNA and L64 0.25% (L64); Pre-treatment with L64 0.25% then mild electrotransfer (L64ET120); Pre-treatment with L64 0.25% then HV (L64HV). With HV 800 V/cm, 0.1 msec.; ET120 8 pulses of 120 V/cm, 20 msec. at 2 Hz. The number of muscle for each treatment was of 10. Statistical significance of the difference relative to injection of free DNA : ** $p < 0.01$, * $p < 0.05$

Fig 4.

Characteristic T1-weighted images of Gd-DOTA distribution in the tibial cranial muscle.

Mouse leg axial section as visualized by MRI at day 3 after injection in the tibial cranial muscle of Gd-DOTA with DNA and pluronic L64 0.25 % (L64 0.25 %), DNA followed by delivery of one pulse 800 V/cm, 0.1 msec. (HV), DNA followed by delivery of 8 pulses, 190 V/cm, 20 msec., 2 Hz (ET) , DNA followed by delivery of 1 pulse 800 V/cm, 0.1 msec. and 0.5 sec later 1 pulse 80 V/cm, 500 msec. (HVLV) and DNA co-injected with pluronic L64 0.25 % followed by delivery of 1 pulse 80 V/cm, 500 msec. (L64LV). The Gd-DOTA trapped in muscle fibers is

visualized by the bright zone near the tibia bone and is indicated by an arrow. On the leg section HVLV approximate position of the two electrodes is indicated by two white bars.

Fig 5.

Effect of pluronic L64 and / or electric field on permeabilization *in vivo*

The distribution of values for each treatment are represented by box plot i.e out of the box smallest and maximum values (10 % and 90 %) then in the box the lower quartile, median and upper quartile.

A Tibial cranial muscle volume permeabilized to the MRI contrast agent Gd-DOTA

The mean value of the volume in μl is given near each box. Group designations on the abscissa are for injection of free DNA (DNA); DNA then HV (HV); DNA then LV (LV); DNA then ET (ET); DNA then HVLV (HVLV); DNA and L64 0.25% (L64); DNA and L64 0.25% then LV (L64LV). With HV 800 V/cm; 0.1 msec., LV 80 V/cm, 500 msec.; HVLV one pulse HV followed 0.5 s later by one LV pulse; ET 8 pulses of 190 V/cm, 20 msec. at 2 Hz; Statistical significance of the difference relative to injection of free DNA : **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$; relative to HVLV: ##### $p < 0.0001$

B Concentration of the MRI contrast agent Gd-DOTA in the tibial cranial muscle volume permeabilized

The mean value of the concentration in nanomoles is given near each box plot. Group designations on the abscissa are for injection of free DNA (DNA); DNA then HV (HV); DNA then LV (LV); DNA then ET (ET); DNA then HVLV (HVLV); DNA and L64 0.25% (L64); DNA and L64 0.25% then LV (L64LV). With HV 800 V/cm; 0.1 msec., LV 80 V/cm, 500 msec.; HVLV one pulse HV followed 0.5 s later by one LV pulse; ET 8 pulses of 190 V/cm, 20 msec. at 2 Hz; There was no significant difference for any group relative to injection of free DNA.

Fig 6.

Correlation between luminescence and volume permeabilized to Gd-DOTA.

Log values of the luminescence and corresponding permeabilized volume to Gd-DOTA of muscle in paired optical imaging and MRI experiments for all the mice studied. Each color corresponds to one treatment. Group designation: for injection of free DNA (DNA); DNA then

Transfection vs permeabilization relationship

HV (HV); DNA then LV (LV); DNA then ET (ET); DNA then HVLV (HVLV); DNA and L64 0.25% (L64); DNA and L64 0.25% then LV (L64LV). With HV 800 V/cm; 0.1 msec., LV 80 V/cm, 500 msec.; HVLV one pulse HV followed 0.5 s later by one LV pulse; ET 8 pulses of 190 V/cm, 20 msec. at 2 Hz.